FURTHER STUDIES ON FEEDING AND DIGESTION IN TRICLAD TURBELLARIA

J. B. JENNINGS

Department of Zoology, The University of Leeds, England

Previous accounts (Jennings, 1957, 1959) have shown that the triclad Turbellaria feed on a variety of invertebrate animals, such as annelids, molluscs, crustaceans and insect larvae, and that the basis of the feeding mechanism is the protrusible plicate pharynx which is thrust through the integument of the prey to withdraw body contents and pass them back in a finely divided condition into the flatworm gut. The penetration of the prey and the subsequent disruption of its tissues appear to be achieved largely by direct muscular action, but the possibility that this is supplemented by some enzymatic activity has not been investigated, apart from a brief study by Westblad (1922), who failed to find digestive activity in extracts of *Dendrocoelum* or *Polycelis* pharynges. On arrival in the gut the food is phagocytosed by columnar cells of the gastrodermis and digested intracellularly. The sequence of food vacuole formation and intracellular digestion has been described in detail (Willier, Hyman and Rifenburgh, 1925; Jennings, 1957, 1959) but little is known of the enzymes concerned, other than the fact that the food vacuoles contain acid phosphatase and leucine aminopeptidase (Rosenbaum and Rolon, 1960).

In the present investigation two species of triclad Turbellaria, one aquatic and one terrestrial, have been investigated by histochemical methods, to locate and identify any enzymes produced by the pharynx to assist its penetration and disorganization of the food. The course of digestion has been similarly investigated, in each species, in an attempt to identify more of the enzymes concerned and to establish the sequence in which they are produced.

Materials and Methods

The two triclad species examined were *Polycelis cornuta* Schmarda (fresh-water) and *Orthodemus terrestris* (terrestrial). The bulk of the work was carried out on the fresh-water species because of its relative abundance and ease of maintenance in the laboratory.

Flatworms starved for 7 days to clear the gut of traces of previous meals were fed on liver, beef fat or starch paste, the two latter foods being made attractive by mixing with frog blood. The foods were heated to 100° C. and subsequently cooled before being presented to the flatworms, to prevent their inherent enzyme activity being confused with any produced within the flatworm pharynx or gut. The flatworms were fixed at progressive intervals up to 48 hours after an observed meal on one or another of the test foods, and serial sections cut at 8 μ examined for enzyme activity in the pharynx, gut lumen and gastrodermis. Full details of the methods used for fixation, preparation of sections and visualization of enzyme activity have

been given in an earlier account of similar studies on digestion in the rhynchocoelan, *Lineus ruber* (Jennings, 1962), and are only summarized here.

Fixation was for 12 hours at 4° C. in 10% formalin buffered to pH 7.0, followed by rapid dehydration in absolute acetone at the same temperature and subsequent embedding in either polyester wax (melting point 37° C.) or paraffin wax (42° C.). When the latter was used, brief clearing in xylol at room temperature was necessary. The polyester technique gave a better histological picture but caused a significant decrease in the demonstrable amount of certain enzymes, notably phosphatases and aminopeptidase, despite the apparent advantage of the lower melting point of the wax, and consequently paraffin wax was used almost exclusively for studies on these particular enzymes. Proteolytic enzymes were demonstrated by the Hess and Pearse (1958) method for endopeptidases of the cathepsin C type (homologous with mammalian chymotrypsin), using as controls incubation media containing cysteine or lead nitrate which act respectively as specific activator or inhibitor, and by the Burstone and Folk (1956) method for exopeptidases of the leucine aminopeptidase type, using heat-inactivated sections as controls. Lipolytic activity was demonstrated after a meal containing beef fat by the Tween 80 method of Gomori (1952), again with heat-inactivated controls. Attempts were made to demonstrate diastatic activity by the Billet and McGee-Russell (1955) method but this gave unsatisfactory results and the presence of carbohydrate-splitting enzymes could only be inferred by tracing progressive digestion of a starch meal by the Lugol's iodine technique. Acid and alkaline phosphatases were visualized by the glycerophosphate methods of Gomori (1952), and controls performed by omitting the substrate from the incubation media and by heat inactivation of sections. The pharynx was examined for possible carbonic anhydrase activity, often associated with production of acid digestive juices, by Hausler's cobalt method (1958), with control sections incubated in the presence of Diamox sodium, a specific inhibitor for this enzyme.

Incubation times at 20° C. and the pH values of the various incubation media can be found in the study on rhynchocoelan digestion referred to earlier.

Observations

The structure of the pharynx and gut

The structure of the triclad pharynx and of the gut and its lining gastrodermis have been described in detail elsewhere (Hyman, 1951; Jennings, 1957). Briefly, in both species investigated here, the pharynx is a highly muscular tube which lies in the pharyngeal chamber in the posterior region of the body. It is directed backwards and can be protruded through the ventral mouth by simple muscular elongation. The pharynx contains along its entire length outer and inner longitudinal and circular muscle layers, a layer of acidophil and basophil gland cells between these, radial muscles and a well developed nerve plexus. The gut proper, in each species, is of the typical triclad pattern with one anterior and two posterior branches, each of which is further subdivided. The gastrodermis consists of a single layer of cells standing on a thin basement membrane and containing only two cell types. The larger and more numerous cells are columnar, 35–40 μ in height, with basal nuclei and granular cytoplasm usually containing phagocytosed food in various stages of digestion. The second type of cell is the "granular club" (Hyman,

1951) or "sphere cell" (Jennings, 1957) and is pear-shaped, 20–30 μ in height, and contains numerous homogeneous spheres which in the fully developed cell are intensely acidophilic and stain strongly with Millon or similar reagents for protein. The spheres within any one cell are always of the same size and appear to mature with the cell. Thus, in small sphere cells the spheres are 1 μ or less in diameter and increase up to 5–6 μ in the mature cell. During prolonged starvation the number of sphere cells decreases, relative to the columnar cells, and the spheres of those persisting show reduced affinity for stains.

Enzymes produced in the pharynx and gut during feeding and digestion

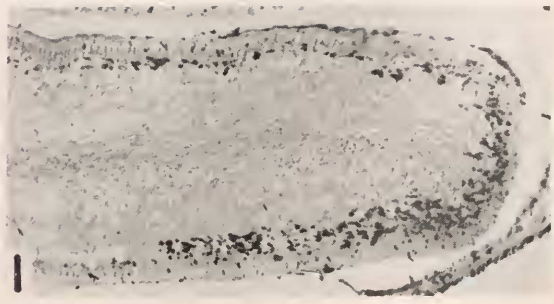
(1) The pharynx

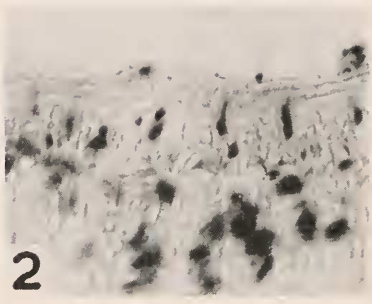
In both *Polycelis* and *Orthodemus* a large proportion of the acidophil gland cells of the pharynx show a strong positive reaction for endopeptidases of the cathepsin C type, particularly around the free distal end (Fig. 1). The glands are flask-shaped and open on to the outer surface, only, of the pharynx (Fig. 2), never into the lumen. Sections of the pharynx prepared immediately after feeding showed that many of these gland cells were discharged and shrunken, and there can be little doubt that their secretions are used to supplement the muscular pressure exerted by the pharynx during the penetration of the prey, by softening or dissolving the tissues of the body wall. The marked concentration of gland cells around the tip of the pharynx supports this conclusion.

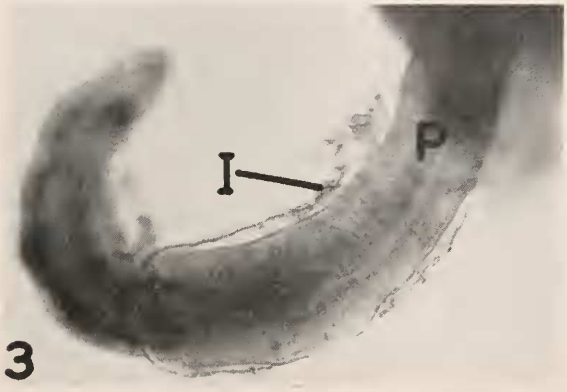
Penetration of the prey occupies 30 to 60 seconds and once within it the pharynx moves about and draws up organs, tissues and body fluid. This part of the feeding process may last for several minutes, and again there can be little doubt that break-up of the prey's body contents by the muscular activity of the pharynx is supplemented by proteolysis effected by secretions from the pharyngeal glands. Since these open on to the outer surface of the pharynx and not into the lumen, their secretions are presumably poured into the body cavity of the prey to attack and disrupt its contents whilst tissues already disorganized are being ingested. In this connection it is significant that the pharynx is always inserted *into* the prey, even when the latter is manifestly small enough to be swallowed whole, as when oligochaetes of a smaller diameter than the resting pharynx are captured. In such cases the pharynx, or its distal portion, is extended until it is slim enough to enter the prey in the usual manner (Fig. 3) and so allow the secretions of the pharyngeal glands to attack its contents. This feeding pattern is followed even with test meals of blood or finely chopped liver, when the pharynx enters the food mass and withdraws material from the center rather than merely being applied to the surface layers.

The optimum pH for visualizing the endopeptidase activity was 5.0, and consequently it was thought that the pharynx might produce acid to provide the proteolytic secretions with the necessary working conditions. The enzyme often concerned with production of acid digestive juices is carbonic anhydrase, but no trace of this enzyme could be found in either the *Polycelis* or *Orthodemus* pharynx.

The cytoplasm of the acidophil endopeptidase gland cells shows at all times a weak reaction for acid phosphatase. No other enzyme activity, proteolytic or otherwise, could be detected in the pharynx of either species.







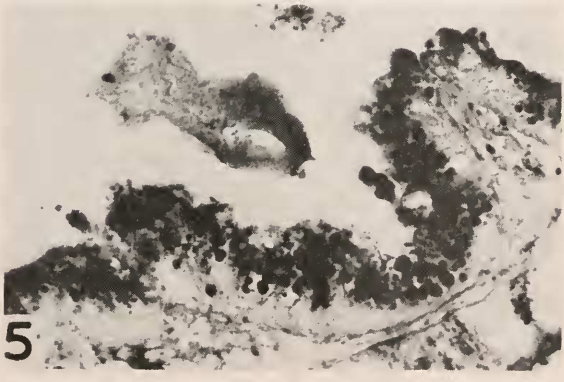


FIGURE 1. Longitudinal section of the *Polycelis* pharynx, showing the distribution of acidophil endopeptidase-producing gland cells. Hess and Pearse method. The tissue at the extreme bottom right is body wall and epidermis, and the dark bodies seen here are rhabdites which have stained strongly with the eosin counterstain. Scale: 1 cm. = 125 μ.

FIGURE 2. A portion of the outer layers of the *Polycelis* pharynx, showing endopeptidase gland cells discharging on to the outer ciliated epithelium. Hess and Pearse method. Scale: 1 cm. = 25 μ.

FIGURE 3. The *Polycelis* pharynx attacking a small oligochaete. Note that the pharynx (P.) has been inserted *into* the oligochaete and that only the integument (I.) remains outside the pharynx. Unstained whole mount. Scale: 1 cm. = 250 μ.

(2) The gut

Cathepsin C type endopeptidases

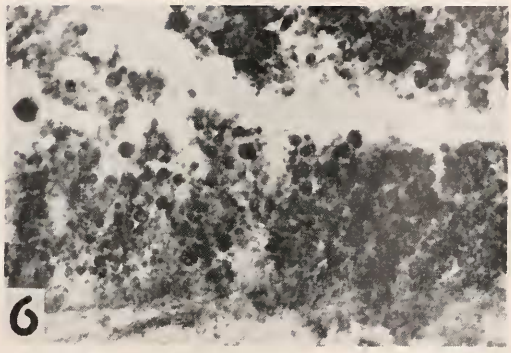
The spheres of the gastrodermal sphere cells show in both species an intense positive reaction for the cathepsin C type endopeptidases (Fig. 4), and a similar reaction is given by fine granules which occur in the cytoplasm of the columnar cells when these are cleared of digesting food by 2 to 3 days' starvation.

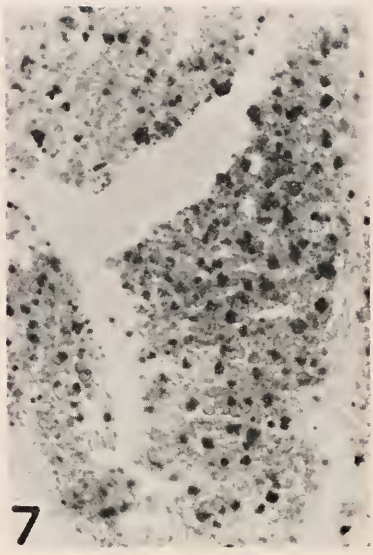
Flatworms killed immediately after a meal of boiled liver show faint traces of endopeptidase activity in the material lying in the gut lumen, and this is derived, no doubt, from secretions poured on to the food by the glands of the pharynx before ingestion. The amount of endopeptidase activity in the contents of the lumen increases with time up to a maximum reached 4 hours after feeding (Fig. 5), and during this time there is a decrease in the number of the large and mature sphere cells relative to the number of columnar cells. This decrease in the number of sphere cells is not constant throughout the gastrodermis, however, and some regions may be quite devoid of them whilst others have the normal complement. Usually the disappearance of sphere cells from a region of the gut coincides with the presence of food and the development of maximum endopeptidase activity in that region, but food showing such activity may be found in parts of the gut lined by the normal proportions of sphere and columnar cells. Such situations are probably due to material in the lumen being passed into a region of the gut away from that where the enzyme activity originated, by the convulsive contractions of the flatworm during fixation. Individual spheres of the same size and reaction as those within mature sphere cells are occasionally found either between columnar cells or lying free in the gut lumen. It would appear from this, and the decrease in sphere cell numbers noted above, that mature sphere cells discharge their contents when food enters the gut, and that the lumen endopeptidase activity comes from this source.

The endopeptidase activity developed in the gut lumen does not cause complete homogenization of the food, and even at the peak of its activity, as shown by the intensity of the histochemical reaction, distinctive components of the food, such as erythrocytes, muscle fibers, liver cell nuclei, etc., are often clearly recognizable. The columnar cells of the gastrodermis commence phagocytosis of the food immediately it enters the gut, and the smaller food particles pass rapidly into the cells so that they are not exposed for long to the lumen proteolysis. The function of the latter appears to be primarily to facilitate phagocytosis by softening and breaking up the larger pieces of the food, rather than to render it completely soluble. There appears to be only an initial discharge of endopeptidase when food enters the gut, not a continuous one for as long as it remains in the lumen, and sections prepared at intervals up to 48 hours after feeding show that any food particles too large for phagocytosis which survive this initial discharge persist unchanged until eventually expelled from the gut. This situation is particularly liable to arise if starved flatworms are allowed to

FIGURE 4. The gastrodermis in a *Polycelis* starved for 7 days, showing sphere cells and columnar cells. The sphere cells show an intense positive endopeptidase reaction. Hess and Pearse method. Scale: 1 cm. = 20 μ.

FIGURE 5. Transverse section of a portion of the *Polycelis* gut 4 hours after a meal of boiled liver. Liver lying in the gut lumen (top left) shows a positive endopeptidase reaction, especially the right-hand portion, and the gastrodermis is loaded with phagocytosed liver showing a similar but stronger reaction. Hess and Pearse method. Scale: 1 cm. = 40 μ.





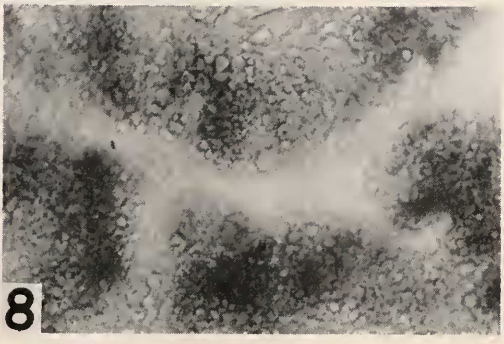


FIGURE 6. Transverse section of a portion of the *Orthodemus* gut 12 hours after a meal of boiled liver. The gastrodermis is loaded with food vacuoles, all showing an intense positive reaction for leucine aminopeptidase. Burstone and Folk method. Scale: 1 cm. = 20 μ.

FIGURE 7. Transverse section of a branch of the *Orthodemus* gut 12 hours after a meal containing a large proportion of fat. Many of the food vacuoles show lipolytic activity, seen here as black spheres or granules. Gomori Tween 80 method. Scale: 1 cm. = 20 μ.

FIGURE 8. Transverse section of the *Polycelis* gut 4 hours after a meal of boiled liver. The cytoplasm and food vacuoles show acid phosphatase activity (dark areas). Gomori method. Scale: 1 cm. = 40 μ.

FIGURE 9. Transverse section of the *Orthodemus* gut as in Figure 6 but treated here for alkaline phosphatase. The food vacuoles show intense alkaline phosphatase activity. Gomori method. Scale: 1 cm. = 20 μ.

feed until replete, when they often ingest more food than can be adequately dealt with in the lumen. The persistence of unchanged food elements in the lumen for up to 48 hours after feeding has been interpreted previously as showing the complete absence of intraluminar digestion (Jennings, 1957), but the present demonstration of endopeptidase activity in the contents of the lumen leaves little doubt as to the occurrence of at least a limited amount of intraluminar digestive activity in the two species investigated here.

Food phagocytosed from the lumen continues to show endopeptidase activity within the vacuoles of the columnar cells, and since this increases in intensity as the vacuoles pass back deeper into the cells, endopeptidases must be secreted into the vacuoles from the surrounding cytoplasm, perhaps from the reactive granules so prominent when the cells are cleared of other inclusions. As the vacuoles pass back into the columnar cells, more form distally until the cells are loaded with food undergoing intracellular digestion and showing intense endopeptidase activity (Fig. 5). Eight to 12 hours after feeding, the contents of the vacuoles are reduced to compact homogeneous masses, and the endopeptidase activity fades gradually, first from vacuoles deep within the cells and then from the rest, indicating that the first stage of digestion affecting breakdown of protein to peptones and polypeptides is completed. The food then passes into the second stage of digestion, in which exopeptidases complete proteolysis down to amino acids, and lipases and carbohydrases attack fats and carbohydrates exposed by the digestion of cell walls or other cytoplasmic membranes.

The optimum pH value for visualizing endopeptidase activity in the lumen and gastrodermis of both species was pH 5.0, and this indication that the first stage of digestion is carried on in an acid medium agrees with the results obtained by feeding test foods plus indicators when a pH value of 4.6 was found in the food vacuoles 6 hours after feeding (Jennings, 1957).

Leucine aminopeptidase (exopeptidase)

Leucine aminopeptidase activity is confined to the columnar cells of the gastrodermis in both species and was never found in either the gut lumen or the sphere cells.

As endopeptidase activity fades from the food vacuoles it is gradually replaced by leucine aminopeptidase, supplemented, presumably, by other exopeptidases not demonstrated by the technique used here. The time of onset of the exopeptidase activity varies with the size of the original meal which influences the amount of food phagocytosed by the columnar cells. Thus, when only a small meal is taken, and each columnar cell forms relatively few food vacuoles, exopeptidase activity appears in the latter as early as two hours after feeding, but when a large meal has allowed the columnar cells to become packed with food vacuoles, the activity may not appear for 12 to 18 hours. On the average, endopeptidase activity is replaced by exopeptidase 8 to 12 hours after feeding.

The exopeptidase activity overlaps the endopeptidase, to a degree determined

FIGURE 10. Longitudinal section through the anterior end of *Polycelis*, showing an eye (E.) and acidophil endopeptidase-producing gland cells (G.C.) in the parenchyma, which discharge through the epidermis. Rhabdites in the latter are stained strongly by the eosin counterstain. Hess and Pearse method. Scale: 1 cm. = 25 μ.

by the amount of food ingested, and recently formed vacuoles in the distal region of the columnar cells may show marked endopeptidase activity whilst those deeper within the cell give a weak exopeptidase reaction. The latter increases in strength with time and appears in more and more of the vacuoles until eventually all food undergoing intracellular digestion gives an intense reaction (Fig. 6). This activity persists for as long as food remains in the gastrodermis and, depending upon the size of the meal taken, may still be present 48 hours after feeding.

Columnar cells of starved flatworms show no reaction for leucine aminopeptidase, and it would appear that exopeptidases, unlike the endopeptidases, are normally present in an inactive form and are not activated until food vacuoles are present.

The optimum pH for visualizing exopeptidase activity was 7.2 in both species, indicating that the second and final stage of proteolysis proceeds in a slightly alkaline medium, as in most other animals.

Lipase

In both species the gastrodermis shows a small amount of lipolytic activity during starvation, and this probably represents the utilization of reserve fat which is laid down in the columnar cells when food is plentiful (Jennings, 1957, 1959).

Both species show lipolytic activity in food vacuoles formed within the columnar cells after a meal containing beef fat (Fig. 7), and this develops as the endopeptidase activity fades. No lipolysis was found in the gut lumen, and pieces of fat too large for phagocytosis lay there quite unchanged until expelled from the gut.

The optimum pH value for demonstrating lipolytic activity was 7.2 in both cases, the same as for the exopeptidase.

Carbohydrases

No conclusive results were obtained from the Billett and McGee-Russell method for β-glucuronidase when applied to sections of flatworms killed at intervals after meals containing boiled starch. Treatment with Lugol's iodine, however, showed progressive conversion and disappearance of the starch within food vacuoles, whilst any remaining in the lumen was quite unchanged. Thus, both *Polycelis* and *Orthodemus* possess diastatic enzymes but these remain as yet unidentified.

Phosphatases

Both acid and alkaline phosphatases occur in the columnar cells of the gastrodermis during intracellular digestion, and there is a marked correlation between the development of endopeptidase and acid phosphatase, in the first stage, and the remaining digestive enzymes and alkaline phosphatase, in the second.

In starved individuals the cytoplasm of the columnar cells shows a weak reaction for acid phosphatase but none for alkaline. As food vacuoles form and endopeptidase activity develops within them, there is a simultaneous but less marked increase in acid phosphatase in both the vacuoles and the surrounding cytoplasm. The peak of acid phosphatase activity coincides with that of the endopeptidase (Fig. 8), but at no time is the reaction particularly intense. This is due, perhaps, to loss of enzyme during preparation of sections, since check sections of mammalian tissue treated in the same way show less than the expected amount of acid phosphatase.

The acid phosphatase activity decreases as the endopeptidases fade from the vacuoles, and is gradually replaced by alkaline phosphatase. This develops in both cytoplasm and vacuoles simultaneously with the leucine aminopeptidase and lipase, and at its peak every vacuole shows a most intense reaction (Fig. 9). The activity persists for as long as food vacuoles are present in the columnar cells.

Neither acid nor alkaline phosphatases are demonstrable in the sphere cells or gut lumen at any stage of digestion.

The pH optima for demonstration of the two phosphatases were, respectively, pH 5.0 (acid) and pH 9.0 (alkaline).

Enzymes in the parenchyma

During starvation, regions of the parenchyma often show a weak reaction for endopeptidase, aminopeptidase and lipase activity. This reflects, no doubt, the utilization of reserve protein and fat which are stored in the parenchyma (Jennings, 1957).

Certain acidophil gland cells which lie in the parenchyma along the anterior margin of the body give a marked endopeptidase reaction (Fig. 10). These glands are of an elongate flask shape and discharge between the epidermal cilia. Their function is unknown, but it is possible that their secretions are passed over the body by ciliary action during locomotion to help in keeping the surface free of micro-organisms or to make the flatworm distasteful to would-be predators.

Discussion

The main features of interest emerging from the present study on triclad feeding and digestion are, respectively, the demonstration of proteolytic activity in the acidophil gland cells of the pharynx and the proof that digestion is not exclusively intracellular as was previously believed.

The presence of cathepsin C type endopeptidases (proteases of the type initiating proteolysis) in the pharynx glands, the concentration of such glands around the tip of the pharynx, and the discharged and shrunken appearance of the glands immediately after feeding leave little doubt that proteolytic secretions are used to supplement muscular action in the penetration and subsequent internal disorganization of the prey by the pharynx. The triclads are not unique amongst the Turbellaria in this respect, however, for the acotylean polyclads likewise use proteolytic secretions from the pharynx or gut to supplement the muscular action of the pharynx during pre-ingestion break-up of the food (Jennings, 1957). In their case the pharynx is of the same basic structure as the triclad but is much expanded to form a ruffled curtain—the ruffled plicate pharynx—which is extended over the prey to envelope it and act as an external "stomach," rather than being inserted into it to act as a suction tube.

The belief that digestion in the triclad is exclusively intracellular rested on the fact that recognizable food elements may persist in the lumen for up to 48 hours after feeding, but whilst the present work has confirmed that this does occur, for reasons mentioned in the text, it has also shown beyond reasonable doubt that there is some intraluminar digestion by endopeptidases.

The endopeptidase responsible for intraluminar digestion is produced by the

sphere cells of the gastrodermis which in the past have been regarded as protein reserve cells (Hyman, 1951; Jennings, 1957). This conclusion was based on the progressive reduction in the number of sphere cells during starvation, but in view of the undoubted glandular nature of these cells this probably represents a simple regression, such as occurs in the cells of other animal digestive organs during prolonged starvation, rather than the utilization of specific protein reserves.

Intraluminar digestion is followed by phagocytosis and completion of digestion intracellularly by exopeptidases, lipase and carbohydrases. This sequence of events closely resembles that occurring during digestion in the related rhynchocoelan, *Lineus ruber* (Jennings, 1962). In the rhynchocoelan, however, lumen digestion is far more extensive and results in the food being completely homogenized before it enters the gut cells. This is clearly related to the fact that the food is swallowed whole, whereas in the triclad it is already considerably broken up when it reaches the gut, and the bulk of it is immediately available for phagocytosis and intracellular digestion. Consequently there is relatively less intraluminar digestion in the triclad, and what does occur appears to be aimed at reducing the particle size of the food to make it available for phagocytosis, rather than at achieving complete breakdown to simpler substances.

The difference in the amount of intraluminar digestion in the triclad and the rhynchocoelan, itself the result of differences in the respective feeding mechanisms, is reflected in the subsequent intracellular processes. In the rhynchocoelan, food entering the gastrodermis passes almost immediately into the second exopeptidase stage of digestion. In the triclad, food may enter the gastrodermis only slightly affected by the lumen-acting endopeptidase, or even completely unaffected, if phagocytosed soon after the meal, and consequently it must first be attacked by endopeptidases before it is available to the later acting enzymes. As a consequence of this there is far more intracellular endopeptidase activity in the triclad than in the rhynchocoelan. This affords a good demonstration of the effect a particular type of feeding mechanism may have upon subsequent digestive processes.

The two types of phosphatase found in the triclad gut appear to be linked with formation of the intracellular enzymes. Acid phosphatase is closely linked with the first or endopeptidase stage, and Rosenbaum and Rolon (1960) suggest that it may be concerned with food vacuole formation. Alkaline phosphatase is linked with the appearance of the later acting enzymes, and may well be concerned in the release of energy needed for secretion of the various enzymes and the absorption of the products of digestion from the vacuoles.

Summary

1. Feeding and digestion in two species of triclad Turbellaria, one aquatic, the other terrestrial, have been investigated by histochemical methods to locate and identify a selection of the enzymes concerned in the two processes.

2. In both species the pharynx possesses acidophil gland cells which produce endopeptidases of the cathepsin C type, and the available evidence indicates that these are used to assist the pharynx in its penetration of the prey's body wall, and the subsequent disruption of the body contents prior to ingestion.

3. Food entering the gut is attacked by extracellularly-acting endopeptidases, similar to those produced in the pharynx, and originating from the sphere cells of

the gastrodermis. This intraluminar digestion continues and extends break-up of the food initiated by the pharynx, and serves to make the bulk of it available for phagocytosis and intracellular digestion.

4. Columnar cells of the gastrodermis phagocytose food from the gut lumen and digest it within vacuoles containing enzymes secreted from the cytoplasm in a definite sequence.

5. The contents of the food vacuoles are attacked first by endopeptidases similar to those secreted into the gut lumen and acting in an acid medium of pH 5.0.

6. Endopeptidase activity within the vacuoles is eventually replaced by exopeptidases, such as leucine aminopeptidase, plus lipase and unidentified carbohydrases, all acting in a slightly alkaline medium of pH 7.2.

7. Secretion of the various intracellular enzymes involves the appearance of phosphatases in both the cytoplasm and the vacuoles of the columnar cells. Acid phosphatase appears to be concerned with the secretion of endopeptidase in the first stage of intracellular digestion and alkaline phosphatase with the production of the other digestive enzymes.

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